The use of PCR in the surveillance, characterization and diagnosis of influenza

Report of the 9th WHO Working Group Meeting on RT-PCR for the Detection and Subtyping of Influenza Viruses

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Contents

Abbreviations and acronyms

AIV	avian influenza virus
APHL	Association of Public Health Laboratories
BPL	beta-propriolactone
CC	collaborating centre
CDC	Centers for Disease Control and Prevention (US)
CEIRS	Centers of Excellence for Influenza Research and Surveillance
CHP	Centre for Health Protection (Hong Kong SAR, China)
CLIA	Clinical Laboratory Improvement Amendments
CLSIS	CDC Sharepoint Site for Laboratory Support for Influenza Surveillance
CoAg	cooperative agreement
Ct	cycle threshold
CVV	candidate vaccine virus
ECDC	European Centre for Disease Prevention and Control
EQA	external quality assessment
EQAP	External Quality Assessment Programme (WHO)
ERLI	European Reference Laboratory Network for Human Influenza
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration (US)
GISRS	Global Influenza Surveillance and Response System (WHO)
GLP	good laboratory practice
HA	haemagglutinin
HI	haemagglutination inhibition
HP	highly pathogenic
HPAI	highly pathogenic avian influenza
IRR	International Reagent Resource (formerly Influenza Reagent Resource)
ISO	International Organization for Standardization
LP	low pathogenic
LPAI	low pathogenic avian influenza
LPHI	local public health institutes
M	matrixNA neuraminidase
NAI	neuraminidase inhibitor
NGS	next-generation sequencing
NIID	National Institute of Infectious Diseases, Japan
NRL	National Reference Laboratory
OFFLU	OIE/FAO Network of Expertise on Animal Influenza
OIE	World Organisation for Animal Health
PAHO	Pan American Health Organization
PCR	polymerase chain reaction
pdm	pandemic
PIP	pandemic influenza preparedness
PT	proficiency test
QA	quality assurance
QC	quality control
RNA	ribonucleic acid
rRT-PCR	real-time reverse transcription polymerase chain reaction
RSV	respiratory syncytial virus
RT-PCR	reverse transcription polymerase chain reaction
SAR	Special Administrative Region
SNP	single neucleotide polymorphism
VCM	Vaccine Composition Meeting
WER	Weekly Epidemiological Record
WHO	World Health Organization
WHO/Europe	WHO Regional Office for Europe

1.0. Introduction

1.1. Background – PCR and the WHO PCR Working Group

Real-time reverse transcription polymerase chain reaction (rRT-PCR) technology has become the established basis of both influenza virologic surveillance and diagnostic activities in a broad range of laboratories that are part of the WHO Global Influenza Surveillance and Response System (GISRS). rRT-PCR is a rapid and sensitive method to detect influenza genetic material in clinical specimens, and for many years it has been the laboratory test of first choice for the rapid detection of influenza viruses with pandemic potential. The emergence of human cases of influenza A(H7N9) virus infections in China in 2013, and the various widely detected A/H5 reassortants – A(H5N6), A(H5N5), A(H5N8) and A(H5N2) – have highlighted the importance of having methods available to rapidly detect and subtype non-seasonal viruses.

To ensure detection of circulating influenza viruses that undergo frequent mutation, it is essential to update rRT-PCR primers and protocols in a timely manner, to maintain the necessary sensitivity and accuracy of the tests. This is particularly evident in the case of influenza A(H5N1), where the emergence of multiple genetic groups and continuous mutation in recent years makes the review, updating and validation of the different primers and protocols even more crucial. At the same time, the wide application of PCR technology for routine influenza surveillance in many countries has created challenges for global virus monitoring and virologic surveillance.

Availability of genetic sequence data, in particular full genome sequence data through the use of high-throughput next-generation sequencing (NGS), presents opportunities and challenges. The use of NGS in the GISRS network, and its usefulness in the timely tracking of influenza virus evolution, helps to answer questions related to response strategies to potential pandemic events based on vaccines and antiviral drugs. There is a growing need for such information. The WHO PCR Working Group[1] (PCR WG) was established to serve as an expert technical group to address these needs for the benefit of the GISRS as a whole.

1.2. Meetings of the PCR WG

The PCR WG held annual meetings at WHO headquarters in Geneva, Switzerland, from its inception in 2007 until 2015; no meeting was held in 2016. The 2015 meeting was held on 23 and 24 June 2015, and the objectives of were to:
- review work performed since the previous meeting;
- improve quality assurance (QA);
- continue collaboration with the World Organization for Animal Health/ Food and Agriculture Organization of the United Nations (OIE/FAO) Network of Expertise on Animal Influenza (OFFLU);
- review current PCR protocols and discuss improvements for publication on the WHO website; and
- discuss new molecular diagnostic technologies for the detection of novel influenza viruses.

Since the 2015 meeting, the following actions have been taken:
- PCR protocols have been updated[2];
- the need for virus isolation has been emphasized;
- the shipment of unsubtypeable influenza A viruses (i.e. viruses that cannot be subtyped because they are novel or low titre) to WHO collaborating centres (CCs) has been emphasized;
- WHO's PCR External Quality Assessment Programme (EQAP)[3] now includes non-seasonal viruses that are not A/H5N1; and
- viruses for antiviral susceptibility testing have continued to be included in the PCR EQAP.

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[1] http://www.who.int/influenza/gisrs_laboratory/pcr_working_group/en/
[2] http://www.who.int/influenza/gisrs_laboratory/molecular_diagnosis/en/
[3] http://www.who.int/influenza/gisrs_laboratory/external_quality_assessment_project/en/

The objectives of this 2017 meeting were to:

- discuss the role of PCR in surveillance and diagnostics;
- update PCR protocols;
- review the activities and input of OFFLU;
- discuss the role of sequencing;
- discuss the role of virus isolation; and
- discuss improvements to QA and quality control (QC) in the context of EQAP and the United States (US) Centers for Disease Control and Prevention, Atlanta, Georgia (CDC) – specifically, the current status, gaps and the way forward.

The expected outcomes of the 2017 meeting were to:
- provide updated protocols on the WHO website;
- publish meeting reports in the Weekly Epidemiological Record (WER) and on the WHO website;
- provide advice on EQAP panel composition; and
- provide guidance on NGS for the GISRS network.

2.0. Updates from WHO CCs and H5 reference laboratories

Representatives from WHO CCs provided general updates on their activities over the previous year. This section summarizes the presentations.

2.1 Dr Yi-Mo Deng, WHO CC for Reference and Research on Influenza, Melbourne, Australia

The main points presented by Dr Deng were as follows:
- a vaccination effectiveness study in Australia resulted in more subtyping and lineage detection using rRT-PCR assays on clinical samples from the Australian general practitioner surveillance network, because many of these samples are not subtyped;
- more NGS was performed at the CC, especially using subtype A(H3N2) viruses, which are difficult to propagate; this work concentrated on the hemagglutinin (HA), neuraminidase (NA) and matrix (M) genes rather than the full genome, to obtain more information about the clade;
- reagent preparation for respiratory syncytial virus (RSV) surveillance was underway;
- a new director, Dr Kanta Subbarao, began at the CC in November 2016; and
- a virus isolation workshop will be organized at the CC in May 2017, to help various regional laboratories to improve their capabilities in virus isolation.

The number of samples submitted to and processed at the CC in 2016 was similar to that of previous years, peaking in September. Most of the samples were submitted from Australia and New Zealand, with others coming mainly from South Pacific countries and South-East Asia. In 2016, most samples were subtype A/H3 viruses.

Between late 2105 and early 2016, the CC detected pandemic (pdm) A(H1N1) 6B.2 and 6B.1 viruses for which the older CDC method for detection of A(H1N1)pdm viruses showed reduced sensitivity owing to a G954A mutation. The probe has now been updated in the latest CDC kit, to allow detection of both variants.

The CC began using NGS in 2014. Over 700 viruses, mainly of the A/H3 subtype, were sequenced by NGS in 2016, compared to 300 viruses that were sequenced by the Sanger method. The number of full viral genomes obtained using NGS in 2016, at just over 140, was almost double the 80 obtained in 2015. Full genomes were obtained from a selection of representative viruses from different regions.

Most of the A(H1N1)pdm viruses sequenced were clade 6B.1. A total of 233 A(H1N1)pdm viruses were sequenced – six were clade 6B, 211 were clade 6B.1 and 16 were clade 6B.2.

Most of the A(H3N2) viruses sequenced were clade 3C.2a1. A total of 794 A(H3N2) viruses were sequenced – 156 were clade 3C.2a, 467 were clade 3C.2a1 and 171 were clade 3C.3a.

The proportions of influenza B viruses sequenced that were of Victoria or Yamagata lineage were similar. Of the 144 influenza B viruses were sequenced, 81 were Victoria lineage and 63 were Yamagata lineage.

Preparations were being made to conduct RSV surveillance, as follows:

- a multiplex rRT-PCR was established to differentiate between RSV-A and RSV-B, using two probes validated through a number of different viruses and the previous EQAP;
- a sequencing protocol for the RSV surface proteins G and F in both RSV-A and RSV-B was established; and
- the CC is in the process of establishing a NGS platform for RSV – the MinION platform may be explored for this application.

2.2 Dr Xiang Zhao, Chinese Center for Disease Control and Protection, Beijing, China

The main points presented by Dr Zhao were as follows:
- in both northern and southern network laboratories, most influenza A viruses were subtype A(H3N2) and A(H1N1)pdm;
- from October 2016 to 2 April 2017, the number of human A(H7N9) cases reported in mainland China was greater than in previous years, at 1340 confirmed cases, 526 of which were fatal:
  - o this is the fifth wave of A(H7N9)infections, and both the number of cases and the mortality rate (39.3%) were greater in this wave than in previous waves;
  - o advanced age and sex were both risk factors, with more cases reported in elderly males;
- between 1 September 2016 and 11 April 2017:
  - o two cases of A(H5N6) infections were reported, both fatal (both were clade 2.3.4.4);
  - o four cases of A(H9N2) infections were reported in children, all of which were of the Y280 clade – three of these four cases were associated with poultry exposure;
- the primers and probes used to detect A(H3N2) viruses were changed because the original primers and probes were no longer effective;
- two digital PCR platforms were used (a Life Technologies QuantStudio 3D and a BioRad QX200); although rRT-PCR remains the primary method used, digital PCR proved useful for determining viral subtypes and copy numbers without an external reference; and
- a rapid rRT-PCR system was also used; employing the taqman method, this system can perform 40 cycles of PCR in 17 minutes.

2.3 Dr Tsutomu Kageyama, National Institute of Infectious Diseases, Tokyo, Japan

Between November 2016 and March 2017, cases of avian influenza virus (AIV) subtype A(H5N6) infection in poultry and wild birds were identified in nearly half of the prefectures in Japan. Hokkaido, Aomori, Gifu, Miyagi and Niigata prefectures saw both poultry and wild bird cases. Chiba, Kumamoto, Miyazaki and Saga prefectures reported poultry cases only; the other 17 prefectures reported cases in wild birds only. A genetic analysis of one of these viruses, A/duck/Hyogo/1/2016 (H5N6) clade 2.3.4.4, was reported. This virus was nominated as a candidate vaccine virus (CVV). It contained a highly pathogenic (HP) multibasic cleavage site in the HA and Q222 and G224 in the receptor binding domain, which is indicative of binding specificity for avian-type alpha 2,3-linked sialic acids. The HA was most closely related to A/environment/Kagoshima/KU-ngr-I/2016 (H5N6), collected in 2016. There were no mutations associated with resistance to NA inhibitors or amantadine, but markers of high virulence in mammals were present at PB2 627 (Glu) and 701 (Asp). A truncation of 24 amino acids was also found in the PB1-F2 protein. All conventional RT-PCR primers and rRT-PCR primers and probes were effective in detecting these A(H5N6) clade 2.3.4.4 viruses.

A new rRT-PCR platform was established for the detection of A()H9N2 viruses. This assay showed a good dynamic range and was effective in detecting the CVVs A/chicken/Hong Kong/G9/1997

(H9N2) and A/Hong Kong/308/2014 (H9N2). It was also effective in detecting recent 2015 A(H9N2) viruses of clade Y280/G9 and G1. Most of the older A(H9N2) viruses could also be detected, although primer mismatches in some cases resulted in decreased sensitivities. There was no evidence of nonspecific reactivity.

The surveillance system and the use of rRT-PCR EQAP in 2016 in Japan were discussed. This system comprises a network of sentinel clinics and hospitals that forward clinical samples to local public health institutes (LPHI) for isolation and PCR amplification. About 5–10% of these samples are then forwarded to Japan's National Institute of Infectious Diseases. The EQAP was implemented in all 74 LPHIs, and comprised samples with either very low or high concentrations (3 and 70 copies/uL, respectively). Viruses included were A(H5N1), A(H1N1)pdm, A(H7N9) and A(H3N2). All LPHIs could detect high-concentration samples, but detection of low-concentration samples was between 92% and 97%, possibly due to primer deterioration. Troubleshooting is currently underway.

2.4 Dr Rodney Daniels, WHO CC for Reference and Research on Influenza, London, United Kingdom

The United Kingdom CC moved to the Crick Institute in October 2016, and expected to have containment level 3 laboratories functional by July 2017. Containment level 4 laboratories came online from mid-February 2017, so the CC can accept A/H5 and A/H7 viruses. At the time of the meeting, the CC had received a A(H5N1) virus from a human case in Egypt and was awaiting receipt of a highly pathogenic avian influenza (HPAI) A(H7N9) virus from China. Viruses from Ghana were also received, one of which was selected as a CVV and sent to St Jude Children's Research Hospital, Memphis, Tennessee, US, for work up. The CC could not accept further west African viruses in January but can accept them now. The CC also received Eurasian swine A(H1N1) viruses from human cases in Italy, the Netherlands and Switzerland; these viruses were under investigation.

Only a small amount of rRT-PCR was performed at the CC because national influenza centres (NICs) were now performing this before shipment. The increased use of rRT-PCR seemed to have resulted in a loss of skills in tissue culture and virus isolation at some NICs, which had recently shared clinical specimens only. The CC had requested that NICs send both clinical specimens and corresponding virus isolates.

The CC now uses MiSeq NGS for most isolates and some clinical samples. Due to the increased use of NGS, the CC is now adopting a "sequencing first" strategy. However, the time delay associated with NGS was a concern. To address this, the CC had set up a collaboration with Oxford Nanopore Technologies to develop MinION RNA sequencing.

The report for the February 2017 vaccine composition meeting (VCM) revealed that 78% of viruses received were subtype H3, clade 3C.2a, with a few clade 3C.3a. The ratio of 3C.2a1 to 3C.2a was 2:1. Of the A(H1N1)pdm viruses, all were clade 6B.1. Most of the influenza B viruses were Victoria lineage, at a ratio of about 2:1 with Yamagata lineage viruses. No Victoria lineage deletion variants had been observed.

2.5 Dr Stephen Lindstrom, WHO CC for the Surveillance, Epidemiology and Control of Influenza, US CDC

Detection of unsubtypeable influenza A viruses were reported to the CDC in March 2016 from three US states. Specimens had low cycle threshold (Ct) values, and sequencing revealed that they were A(H1N1)pdm viruses belonging to clade 6B.1. Some viruses had three mutations in the probe region, which resulted in failure to determine the subtype. An announcement was sent out asking for such viruses to be sent to the CDC, after which about 36 cases from 11 different states were reported. Overall, the prevalence of these viruses never exceeded 2% and did not increase in circulation. Although viruses with two mutations in this region could be subtyped, the third mutation caused problems with this assay. Because the location of the third mutation was variable, no single assay update could accommodate these mutations. Hence, probes were updated based on two

mutations, because the probes were long enough to accommodate the third mutation. This is the first time that the CDC has updated an existing assay with the Food and Drug Administration (FDA). Validation testing included limit of detection for range finding (using 5 and 20 replicates), analytical reactivity for inclusivity only, limited clinical performance to show reactivity against more than 50 negative specimens and more than 30 positive specimens, and testing for reproducibility and precision. The updated subtyping kit was called CDC Influenza A/Subtyping Kit (VER2). Following this update, the pre-2009 A/H1 assay was retired, the positive control was updated from *pooled* influenza positive control to *seasonal* influenza positive control, and the ZEN quenched probes were qualified to demonstrate performance to the FDA.

The Influenza Reagent Resource is still available for the ordering of reagents, but has been renamed the International Reagent Resource (IRR).[4] The change in name reflects the move to make the IRR more inclusive for reagents outside of influenza.

CDC influenza rRT-PCR kits were distributed to 117 countries. About 600 kits per year were distributed in 2014 and 2015, and 836 were distributed in 2016. This increase over previous years was mainly due to the introduction of the VER2 subtyping kit, expiration of the A/H5 kit and increased distribution of influenza B genotyping kit. As of 4 April 2017, 219 kits had been distributed in 2017.

The CDC Sharepoint Site for Laboratory Support for Influenza Surveillance[5] contains information on protocols and procedures for public health laboratories. Currently, 212 registrants from 90 countries are registered. Users have been advised to check their status on the system, because if the registrant has not used the site for a period of time they will be purged, requiring them to re-register.

Other potential pandemic viruses in the US were discussed. In swine, two genotypes of A(H3N2) variant virus (A(H3N2)v) and four genotypes of A/H1 variant viruses work observed. In avian hosts, there were both HP and low pathogenic (LP) North American subtype A/H7 viruses, although LP viruses predominated. In canine hosts, there were A(H3N2) viruses of Asian avian origin and A(H3N8) viruses of equine origin. In feline hosts, LP North American A(H7N2) viruses of avian origin were detected in December 2016 and caused one known human infection. The detection of swine and avian viruses is important during the spring season because of the movement of animals. Swine origin A(H3)v and A(H1)v cases are seen sporadically in humans, raising the question of whether these viruses need to be shared in EQAPs.

In discussing rRT-PCR platforms, it was noted that the likelihood of discontinuation of a machine should be considered in providing platform advice to NICs. For example, the Thermo 7500 Fast was cleared by the FDA in 2008 and will be maintained until 2022, but is now considered a legacy instrument. For FDA clearance, the CDC is qualifying protocols on platforms that already have such clearance. These platforms are the Thermo ABI QuantStudio Dx (QSDX) and the QIAGEN RotorGene Dx (QMDX), which include options for locked results analysis and reporting. Several nucleic acid extraction machines are being discontinued: EasyMag (BioMerieux), and the Magnapure Compact and LC (Roche). After vetting, the QIAGEN EZ1 Advanced XL and the Roche Magnapure 96 were selected as candidates for FDA clearance and are currently under evaluation.

2.6 Dr John Franks, WHO CC for Studies on the Ecology of Influenza in Animals, St Jude Children's Research Hospital, Memphis, Tennessee, US

The Centers of Excellence for Influenza Research and Surveillance (CEIRS) QA/QC panels were discussed; these panels have been ongoing since 2012. They are offered twice a year, and CEIRS laboratories are required to participate at least annually. Non-CEIRS laboratories can also request the panels. The first panel for 2017 has been sent out, and comprises different avian and mammalian panels. The mammalian panels contained only swine viruses, and the avian molecular panel was heavily biased to North American viruses. Some non-standard subtypes that CDC kits

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[4] See https://www.internationalreagentresource.org/
[5] See www.CDC.gov/flu/clsis

do not detect were included, such as A/H4 and A/H6 viruses. Results are based on the ability to detect influenza A viruses; viral subtyping is optional and is not scored. Previous results showed that about 80% of individuals passed these panels. Poorly performing laboratories tend to have a high turnover of technicians and thus continue to do poorly. Those individuals that scored 100% on the mammalian panel also reported correct subtype results at a high rate (88%). In the serology panel, most laboratories were using IDEXX kits and a minority were using haemagglutination inhibition (HI) subtyping assays.

St Jude is currently using the available primer probe sequences verified by the CDC for most surveillance activities, with the exception of the North American swine surveillance team, which screens samples for influenza A viruses with a custom, multitarget H1/H3, N1/N2 design offered through Life Technologies.

2.7 Dr Janice Lo, H5 Reference Laboratory, Department of Health, Hong Kong SAR, China
Dr Lo presented an update on the activities of the diagnostic laboratory:

- The laboratory screens respiratory specimens by PCR for a panel of respiratory viruses, including influenza viruses. About 4000 specimens are screened weekly. At the time of the meeting in 2017, three cases of A/H7 had been detected, whereas A/H5 and A/H9 viruses had not been detected.
- The Flu Express publication was discussed. This publication is uploaded weekly by the Surveillance and Epidemiology Branch, and contains sentinel and laboratory surveillance data.
- Influenza activity in Hong Kong SAR this season has been low, mainly consisting of A/H3 viruses.
- Since February 2014, the laboratory has transitioned from primarily conducting virus isolation to primarily conducting molecular detection. This has resulted in an increase in sensitivity of about 10%.
- In longitudinal samples obtained from patients, PCR can show a "lingering" of influenza genes after virus can no longer be isolated. Therefore, patient monitoring on a daily basis may not be of much value.
- A specific case reported on 3/3/2017 showed strong positivity for influenza A but was negative by H7 subtyping and positive for N9. Sequencing revealed three mutations in the H7 specific probe area. A new probe was designed to better detect viruses containing these mutations. Hence, when the transition to molecular testing was made, parallel virus isolation was maintained for samples from outbreaks or from severe infections from the intensive care unit.

2.8 Dr Leo Poon, Center for Influenza Research, the University of Hong Kong, Hong Kong SAR, China
An annual 2-week long virology course was conducted at the University of Hong Kong (HKU)-Pasteur Research Pole. The course included hands-on training in RT-PCR and virus culture, using influenza as an example.

Influenza surveillance activities were conducted in wild birds at the MaiPo nature reserve, in terrestrial poultry at wet markets, in swine at slaughter houses and in humans in community studies. Surveillance in wild birds comprised about 4000 samples per season, both from birds and from the environment. Viruses in environmental samples were isolated in eggs, followed by typing and subtyping by RT-PCR. All samples were LP; however, in November 2016, HP A(H5N6) clade 2.3.4.4 viruses were detected in northern pintails at MaiPo nature reserve on two sampling occasions. These viruses were similar to a wild bird virus isolated in Korea.

Surveillance for AIV in poultry in Hong Kong before 2017 involved virus isolation in eggs followed by HA and HI assays and RT-PCR for typing and subtyping. About 400 samples per month, covering wholesale and retail markets, were analysed to assess the effectiveness of virus control measures.

A single isolation of an A(H7N9)virus in an environmental sample was obtained from a wet market.

Based on concerns surrounding A(H7N9), the detection algorithm was changed to speed up reporting to the government. The new algorithm involves RT-PCR on environmental samples for M, H5 and H7 in parallel, before virus isolation in eggs for M gene positive samples, and HA and HI assays.

Surveillance for influenza viruses in swine involved the collection of 500–600 samples per month, comprising mainly oral and nasal swabs obtained from carcasses. Swabs were used for virus isolation in eggs and in Madin Darby canine kidney cells, followed by PCR genotyping and subtyping, and genomic sequencing using NGS. The isolation rate was about 1%, and the most commonly isolated viruses were A(H1N1)pdm, A(H1N1) containing Eurasian HA and NA, A(H1N2) containing Eurasian HA and human NA, and A(H3N2) containing human HA and NA. The overall impression was that these viruses were reassorting with each other, giving rise to new genotypes.

Human surveillance was conducted on household and vaccine cohorts, using standard WHO protocols for RT-PCR assays to detect influenza A (seasonal A/H1, A(H1N1)pdm and seasonal A/H3) and influenza B (Yamagata and Victoria lineages). About 3000 samples from the community studies were screened.

2.9 Dr Isabella Monne, Istituto Zooprofilattico Sperimentale delle Venezie, Padua, Italy
Dr Monne presented an overview of the challenges in the animal world and recent developments in diagnostic approaches.

Since the end of 2016, an increase in new influenza cases has been reported both in poultry and wild birds. This has occurred mainly as a consequence of the spread of HP H5 clade 2.3.4.4 viruses, although multiple subtypes are currently circulating. From October 2015 to September 2016, 560 cases were reported; in contrast, between October 2016 and March 2017, 2255 cases were reported. As of March 2017, ongoing outbreaks of HP A/H5 and A/H7 viruses have resulted in the destruction of 141 poultry in the Middle East, 5056 in Africa, 73 300 in the Americas, 1 883 976 in Asia and the Pacific, and 4 892 990 in Europe. There was also significant activity of LP A/H5 and A/H7 viruses.

The co-circulation of multiple HPAI virus subtypes posed challenges for molecular diagnostics. Because of the potentially far-reaching consequences of the detection of certain influenza A virus subtypes and A/H5 goose/Guangdong clades, it is important to have a rapid and reliable diagnostic subtyping assay. The co-circulation of Eurasian viruses in different hemispheres and the co-circulation of HP viruses with LP precursors also pose challenges for detection. For example, the introduction of H5Nx viruses into Italy in late 2016 resulted in detection rates three times higher than normal. Situations such as these highlight the need for flexible diagnostic flow.

The Riems Influenza Typing Assay (RITA) for the detection of multiple subtypes was discussed. It is a subtyping tool that uses rRT-PCR for rapid and reliable direct subtyping of influenza A viruses from original sample material obtained from avian and mammalian hosts. The RITA allows differentiation of 14 HA and 9 NA subtypes.

To reduce time to diagnosis and take into account that sequencing is not always possible at some regional laboratories, a new rRT-PCR assay is being developed based on pathotype and clade-specific probes. Five distinct probes have been developed to pathotype Eurasian A/H5 viruses and detect clade 2.2.1.2, 2.3.2.1, 2.3.4.4a and 2.3.4.4b viruses.

3.0 QA

3.1 EQAP: observations on progress made and future plans
The objectives of QA for EQAP are to:
- maintain the WHO EQAP for the detection of influenza A virus subtypes and type B viruses by rRT-PCR among participants, to monitor quality and standards of performance;
- continue the option of voluntary testing of viruses with reduced susceptibility to neuraminidase

inhibitors (NAIs); and
- promote good laboratory practices (GLPs).

The milestones of the EQAP were as follows:

- 2007 to 2009 (Panels 1–5) – included influenza A/H1, A/H3 and A/H5 and 10 RNA samples per dispatch;
- 2009 (Panel 6) – included A(H1N1)pdm;
- 2010 (Panel 7) – included influenza B (Victoria lineage) and a GLP survey, and was accredited as a proficiency testing provider according to International Organization for Standardization (ISO) 17043 (*Conformity assessment – general requirements for proficiency testing*);
- 2010 (Panel 8) – included four H5N1, H1N1 and H1N1pdm09
- 2011 (Panel 9) – included two gamma-ray inactivated virus samples to test RNA extraction (12 samples total);
- 2011 (Panel 10) – included influenza B (Yamagata lineage) and removed previous seasonal influenza A/H1; the frequency of the panel was subsequently reduced to one per year;
- 2012 (Panel 11) – all viruses were gamma-ray inactivated (10 samples) and included an A/H9 sample and a GLP survey;
- 2013 (Panel 12) – included all beta-propriolactone (BPL) inactivated viruses (10 samples) and A(H1N1)pdm NAI susceptibility testing;
- 2014 (Panel 13) – included all Triton X-100 inactivated viruses (10 samples), added A/H7 and A(H1N1)pdm NAI susceptibility testing, and a sample with mixed 275 H/Y;
- 2015 (Panel 14) – included A(H1N1)pdm NAI susceptibility testing and added testing by a phenotypic method;
- 2016 (Panel 15) – included a GLP survey; and
- 2017 (Panel 16) – ongoing.

Panel results are published in January in the WER. Invitations are sent at the beginning of the year. In 2016, for Panel 15, 184 laboratories from 144 countries were invited. Of these, 177 laboratories in 140 counties participated (three laboratories did not receive the panel due to importation problems). A total of 151 laboratories reported results on time (some of the remaining laboratories requested extended deadlines).

Panel 15 comprises four A/H5 samples, a clade 2.3.2.1 and a clade 2.3.4.4 virus at two different concentrations, an A(H1N1)pdm virus at two different concentrations, an A(H3N2) virus, an A(H9N2) virus, a B (Yamagata lineage) virus and a negative control. Of the 151 reporting laboratories, 87% reported 100% correct results overall and 93% reported 100% correct results for A/H5 viruses. Of the 151 participating laboratories, 96 (63.6%) used manual nucleic acid extraction methodologies and 54 (35.8%) used automated methods. One laboratory used both manual and automated methods. There was no statistically significant correlation between the extraction method used and the results obtained. Three laboratories used conventional RT-PCR only, whereas the remaining laboratories used rRT-PCR with or without conventional RT-PCR. Of the incorrect results obtained using A/H5 samples, six of 14 were obtained from samples of the lowest concentration. Five laboratories reported samples as unsubtypeable or negative. Of these five laboratories, two performed conventional RT-PCR, one used an assay with mismatches in primer and probe sequences, and two reported generally high Ct values (>30) using automated extraction methods for all panel samples. One incorrect result was reported for the sample with the highest A/H5 concentration, which may have been the result of contamination from the adjacent A(H1N1)pdm sample. A/H9 samples proved more problematic than the A/H5 samples. Specific detection was reported from 52 (34.4%) of the 151 laboratories, and the rate of correct identification was 92.7% (140 of 151 laboratories). Among the 11 incorrect results, six laboratories performed specific A/H9 testing, one used a conventional RT-PCR assay with primer mismatches and one used a commercial microarray assay. Of the five laboratories not performing H9 subtyping, two reported other influenza A subtypes and three reported an influenza negative result.

The results of the GLP survey were presented. This was the third such survey, following those in 2010 and 2012, and it had a 75.7% response rate (134 of 177 laboratories). The results showed:

- correlation of availability (or lack of availability) of training opportunities with 100% correct performance (73.4% for availability versus 40.0% for lack of availability) – this finding approached significance (Chi-square with Yates correction, P=0.061);
- more laboratories (76.1%) indicated having a contingency plan in case of equipment failure compared with 2012 (62.2%);
- more laboratories adopted guidelines for the preparation or validation of reagents and controls compared with 2012 (81.3% versus 60.8%);
- more laboratories employed an electronic database system for specimen registration compared with 2012 (93.2% versus 82.5%);
- more laboratories have documented criteria for proper specimen labelling (94.0%), proper transportation and storage conditions (88.1%), and specimen leakage (85.1%) compared with 2012, when only half of the responding laboratories reported having such criteria;
- correlation of available documented nucleic acid extraction procedures (130/134) or otherwise with EQAP performance (72.3% versus 25.0% all correct results) approached statistical significance (Fisher exact test, P=0.074);
- counter-checking was consistently implemented by 71.6% of laboratories and authorization by designated persons (to minimize transcription errors) was consistently implemented by 87.3%;
- the proportion of laboratories that had attained, or were in the process of attaining, accreditation based on standards such as ISO 15189/17025 and those of the College of American Pathologists increased from 39.8% in 2012 to 53.0% in 2016;
- correlation between laboratory accreditation and EQAP performance approached statistical significance (Chi-square test, P=0.076);
- the proportion of laboratories carrying out reporting and documentation of non-conformity events increased by 26.7% (to reach 82.8% in 2016).

3.2 Experience and lessons learned from the US CDC

The CDC provides performance evaluation panels for domestic state public health laboratories. The laboratories receive CDC PCR reagents twice per year to help them meet Clinical Laboratory Improvement Amendments (CLIA) requirements. About 100 kits are sent out on each occasion.

The International Molecular Quality Assessment Panel was first offered in 2011 to influenza cooperative agreement (CoAg) countries, to identify the molecular testing capabilities and needs of countries that have CoAgs with CDC. Countries that have NICs receive research-use only CDC kits for testing specimens. The performance of individual laboratories was assessed based on the ability to detect and correctly identify the included viruses. Information was used to address technical issues, including RNA recovery, rRT-PCR assay issues and platform-specific data analysis issues. This has helped to provide a better understanding of the instruments and testing algorithms in use. The panels comprised 10 BPL-inactivated mock human specimens in an A549 human cell background, to imitate human respiratory specimens. Simulated specimens included A(H3N2), A(H3N2)v, A(H1N1)pdm, A(H5N1), A(H7N9), influenza B samples and negative samples. In 2014, 51 countries participated, including 19 African countries. The samples were similar viruses to those in the EQAP. The proportion of laboratories that achieved at least 90% correct results, which is sufficient to meet CLIA requirements, increased from 77% in 2011, to 86% in 2014, to 94% in 2016, although the 2016 results are still being evaluated. Most laboratories used enzyme chemistries from Invitrogen (47%), followed by Ambion (39%), most of these chemistries are legacy; hence, newer enzyme chemistries must be evaluated. ABI instruments were the most commonly used at 45%, followed by BioRad at 20%. The QIAGEN QIAamp viral RNA extraction methods were the most commonly used at 71%.

Problems encountered were generally not associated with the assays, but rather were due to mistakes in record keeping, such as transcription errors (e.g. reporting Ct values for the wrong primer or probe target), final results interpretation (e.g. misinterpretation of results), and specimen handling and processing (e.g. mislabelling of processed samples).

Two CDC/Association of Public Health Laboratories (APHL) international advanced influenza rRT-PCR workshops were held – one in January 2015 in Antananarivo, Madagascar that involved 16 African countries, and one in March 2015 at the CDC in Atlanta that involved 16 CoAg countries. The 2016 performance evaluation of countries that attended the workshop is underway. Interim results revealed that 55 of 72 (76%) CoAg countries were reporting. It is hoped to achieve a reporting rate of at least 85%.

3.3 Experience and lessons learned from Europe

The results of the 2015/2016 European Influenza Surveillance Network–WHO Regional EQA were presented. The EQAP panel was designed by Public Health England, through network coordination by the European Reference Laboratory Network for Human Influenza (ERLI-Net); pre-testing was performed by the WHO CC, London and the NIC in Lyon, France. Data were collected from 52 participating laboratories online, and were managed by Quality Control for Molecular Diagnostics.[6] The panel contained eight samples of influenza A or B viruses of various concentrations, and one negative sample. The objectives were to assess:

- the rapid detection by PCR or other tests within a defined reporting time frame, including typing and subtyping;
- virus isolation within a defined reporting time frame;
- virus typing after virus isolation using HI or PCR;
- influenza A virus subtyping and influenza B virus lineage determination after virus isolation using HI or PCR; and
- virus strain identification by HI and/ or sequencing.

For the eight samples, 48 laboratories (92%) reported 8/8 correct results, three (6%) reported 7/8 correct results and one (2%) reported 6/8 correct results. These results were an improvement over 2013, where 80% of laboratories reported no errors. The virus isolation test revealed that 36 of 45 laboratories reported no errors (80%), and four laboratories (9%) reported multiple errors. Most laboratories performed antigenic characterization, and errors associated with these tests occurred mainly with A(H3N2) viruses, especially subclade 3c.2a. Genetic virus characterization yielded more accurate results. Based on these results, the WHO Regional Office for Europe (WHO/Europe) developed a regional corrective action plan that included individual training for laboratories showing poor performance and two ERLI-Net training sessions during the 2015/2016 influenza season.

3.4 OFFLU strategy on external QA

The OFFLU laboratory network comprises OIE/FAO reference centres for avian, equine and swine influenza, and other OFFLU-affiliated national veterinary influenza laboratories in strategic locations. The OFFLU proficiency test (PT) is currently focused on AIV diagnostics, and the global OFFLU PT for AIVs is run in parallel to regional PTs. The initial objectives of the OFFLU global PTs were to test and document the ability to detect and characterize AIVs from the Eurasian and American hemispheres; and to assess the diagnostic capability on the same sample(s) across the key OFFLU reference laboratories, using their respective pipelines. A major issue identified was the detection of viruses from distinct hemispheres; this was also seen in regional laboratories, where additional problems included difficulties in accessing sequencing facilities. Despite these findings, there have been substantial improvements in subtyping since 2013.

The OFFLU global PTs were not conducted in 2015 and 2016 due to changes that have caused some complications (e.g. the involvement of a large number of laboratories). Therefore, the final aim of the OFFLU global PT will need to be re-evaluated. The circulation of HP A/H5 viruses in the US in 2015 also caused complications for the US laboratory involved in the organization of the PT. The next PT, in August 2017, will be organized by the Australian Animal Health Laboratory; it will include HP and LP H5 viruses, and additional subtypes such as H9. One panel will be provided every year from 11 laboratories with rotating responsibility. The possibility of sharing the responsibility for these PTs between the WHO and OFFLU was also raised.

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[6] See http://www.qcmd.org/

3.5 Purpose of the EQAP

The general overall purpose of the EQAP and PTs was discussed. The current turnaround time is 7 days for detection and 28 days for virus characterization. Failure to meet the 28-day turnaround generally indicates problems in the laboratory. Overall, it was agreed that 28 days was a reasonable turnaround time. The composition of the EQAP panels was also discussed. When first established, the focus was on A/H5 viruses because of the pressing need for detection capacity. Now, the situation has changed, and the function of the EQAP is to assess laboratory capacity for detection of seasonal and emerging (zoonotic) viruses. It may no longer be necessary to include four A/H5 viruses; instead, the inclusion of other viruses of concern (e.g. A/H7 viruses that continue to infect humans and A/H3v swine viruses) should be considered. Those viruses infecting humans should be prioritized, but beyond that, the EQAP should provide confidence that it can detect non-seasonal influenza viruses that could infect humans. It was agreed that the scoring scheme should be revised accordingly. Ideally, the composition of the next panel should be decided by only a few advisors on an as-necessary basis.

It was agreed that including a GLP survey in the EQAP is valuable because it asks detailed questions relating to laboratory diagnostics that are not included elsewhere. In many Pan American Health Organization (PAHO) countries, Spanish is commonly spoken; therefore, the PAHO Regional Office will be asked to translate the survey into Spanish when needed. Russian is also important for countries of the former USSR. Further discussions regarding this are needed.

The frequency of the survey was also discussed. As an example, in the WHO European Region, GLP survey results are received in September or October and then evaluated. However, because the budget cycle for the following year ends in October or November, it is difficult to find funding for specific activities in the next year. Budget cycling of not more than 2–3 years was recommended, to ensure sufficient time to implement corrective actions if needed. It was also suggested that laboratories would benefit from feedback following the survey; such feedback could be provided in the summary report published in the WER. Publication of a review of the activities of the PCR WG in a peer review journal was also proposed.

The role of NICs with respect to unsubtypeable influenza A viruses was discussed. The terms of reference for NICs state that unsubtypeable viruses should be sent to a WHO CC for further study. However, a significant number of unsubtypeable influenza viruses that infect humans may not be detected using current protocols. Should NICs therefore amend their protocols to detect these viruses, which will be sent to the designated CC? In reality, countries want more capacity, and this core capacity building is also defined in the International Heath Regulations (IHR) (WHO, 2005), which states that countries that have the capacity to detect a new event should also confirm that event. The WG can work to promote, educate and coordinate NICs to ensure that they are compliant with the IHR statement, and can also stress the need for virus sharing. The primary requirement of the NICs should be to differentiate seasonal and non-seasonal viruses, and then identify non-seasonal viruses, particularly those infecting humans.

4.0 PCR protocols for GISRS

4.1 Overview of current PCR protocols for GISRS

The 2015 update of the WHO information for molecular diagnosis of influenza virus (WHO, 2015) was discussed. This document contains three annexes the right information on conventional RT-PCR protocols (Annex 1), rRT-PCR protocols (Annex 2) and sequencing protocols (Annex 3). It also deals with the following with respect to these protocols:

- *validation* – all protocols used should be validated in each laboratory to ensure adequate specificity and sensitivity;
- *QA* – standard QA protocols that include the use of appropriate controls and GLP should be in place; participation in NIC evaluation exercises is highly recommended to confirm that laboratories are achieving an adequate level of sensitivity and specificity in their tests;
- *training of personnel* – familiarity with protocols and experience in correct interpretation of

results are cornerstones for successful execution of the diagnostic tests;

- *facilities and handling areas* – specimen and reagent handling facilities (including cold chains), with appropriate separation for different steps of rRT-PCR, must be in place to prevent cross-contamination; facilities and equipment should meet the appropriate biosafety level; and rRT-PCR should be performed in a space that is separate from that used for virus isolation techniques; and
- *equipment* – equipment should be used and maintained according to the manufacturer's recommendations.

4.2 Gaps and actions relating to GISRS rRT-PCR protocols

Protocols should be updated in a timely manner, and WHO should be alerted to changes so that the network can be informed. Since A/H10 viruses are quite rare and are unlikely to become more prevalent, protocols relating to A/H10 could be removed. Also, given that little feedback is obtained about protocol use or effectiveness, a mechanism to provide feedback would be useful, perhaps as part of the information sheets attached to the EQAP for submission of results.

5.0 NGS and other emerging molecular technologies

5.1 Use of NGS in specific institutions

Information was presented on the use of NGS in different laboratories of the GISRS. The presentations are summarized below.

5.1.1 US CDC

Ongoing activities at the US CDC in relation to NGS are mainly based on Illumina. The front end of the process has been optimized to improve the handling of large datasets. The CDC is working with partners in US state public health laboratories that propagate viruses and send them to the CDC. In this context, NGS is used to obtain sequences at sites receiving specimens that have already been identified as influenza by rRT-PCR. Laboratories share datasets via the cloud; these datasets are then combined for analysis at the CDC using an analysis pipeline. The CDC is also working with Thermo, which supplies FDA approved equipment and software. The aim is to adapt the current pipeline for diagnostic sequencing so that it can be used as a true clinical diagnostic platform, where results are analysed and reported without any user interpretation. One problem associated with this approach is that manufacturers do not have pipelines applicable for FDA clearance for infectious diseases.

5.1.2 Chinese Center for Disease Control and Protection, Beijing, China

NGS capability was developed to allow the timely identification of human cases of the novel A(H7N9) viruses (Bao et al., 2013). Full viral genomes can be obtained in 24 hours, and detection of single nucleotide polymorphisms (SNPs) comprising 1–5% of the viral population can be detected. Two platforms were being used; PGM/S5, which takes 2–4 hours to complete a run, was used during the outbreak; and MiSeq/Miniseq, which takes 2 days to complete a run, was used for routine monitoring. NGS (IonPGM Sequencing 200 Kit ver2) was used to obtain full genomes of human cases of A(H5N6) infection and during the A(H10N8) outbreak in China (Chen et al., 2014). All viruses received by the CNIC are now sequenced by NGS. The "Flucloud" system that is now being used was introduced as a cloud-based data analysis platform.

5.1.3. WHO CC for Reference and Research on Influenza, London, United Kingdom

The CC is not an NIC, and samples do not arrive in a timely manner that would allow diagnostic analysis. The samples received are mainly isolates, although clinical specimens and corresponding virus isolates are often received as a pair. The CC is using Illumina MiSeq platforms, located in the Francis Crick Institute's Advanced Sequencing Science Technology Platform. One run per week is performed (one 96-well plate), followed by analysis using an in-house system that is not cloud based. These RT-PCR amplifications are based on the NGS primers developed by Zhou and colleagues (Zhou et al., 2009; Zhou et al., 2014), using mixes of eight influenza A primers and six influenza B primers. Sequences are available on request.

5.1.4. WHO CC for Reference and Research on Influenza. Melbourne, Australia

The Australian CC is also not an NIC; therefore, fresh, original specimens are not received, and most work is based on isolates. The Ion Torrent PGM is being used at the centre, with multiple protocols for influenza gene amplification. Influenza A and B full genomes were obtained using the Zhou protocol with modifications, and a protocol to sequence influenza A and B HA and NA genes (including M in the case of influenza A viruses) was established in collaboration with Zhou and Wentworth.

An influenza A/B multiplex RT-PCR method for NGS was developed, with the aim of achieving a high-throughput platform for the universal amplification of important genes from diverse epidemic influenza strains. The method uses a mixture of influenza A HA/NA/M and influenza B HA/NA primers in a single PCR reaction. It has proven to be sensitive and robust, and has been validated with thousands of seasonal influenza A and B positive specimens or isolates at several laboratories. An NGS analysis pipeline, FluLINE, has also been developed in collaboration with Duke University, Singapore. FluLINE enables sequence analysis of 96 samples within 12 hours. Automation of NGS library preparation has also been developed using the EpMotion 5075 robot from Eppendorf. This process does not save time but it does save on labour, and minimizes operational variation and potential errors.

5.1.5 St Jude Children's Research Hospital, Memphis, Tennessee, US

In an effort to make use of capacity and promote collaboration in the CEIRS network, the Data Processing and Coordinating Center at the National Institutes of Health (NIH) set up a request for a service network for NGS. Samples are mailed in and results are typically received within 1–2 weeks. Illumina HiSeq and MiSeq are currently the only NGS performed at the centre, yielding about 400 base pair fragments and costing about US $800 per run plus US $50 per sample. A new in-house pipeline is also being developed for dealing with difficult samples and mixed infections. Once this pipeline is running, the goal is to make it available online.

5.1.6 National Institute of Infectious Diseases, Tokyo, Japan

NGS is used at the institute for sequencing but not for diagnosis. Illumina MiSeq is currently being used and analysis is being performed using a CLC Workbench. Library preparation for MiSeq from clinical specimens involves the following; (1) amplification of viral RNA extracted from the clinical specimen by RT-PCR, (2) DNA fragmentation using fragmentase and adaptor ligation using the NEB Next Ultra II DNA Library Prep Kit, and (3) PCR enrichment and inclusion of index sequences. Library preparation for MiSeq from isolated viruses involves the following: (1) RNA fragmentation using the NEB Next Ultra II RNA Library Prep Kit, (2) first and second strand cDNA synthesis, (3) adaptor ligation, and (4) enrichment by PCR and inclusion of index sequences. A cost comparison between MiSeq and Sanger sequencing revealed that, when more than seven samples are sequenced using MiSeq at one time, the cost of one sample is less than if the samples were sequenced by the Sanger method.

5.1.7 Center for Influenza Research, Hong Kong University, Hong Kong SAR, China

NGS is used at the centre for research only. It proved particularly useful in the household transmission study. In this study, samples obtained from a single household were almost identical in Sanger sequencing, whereas NGS revealed much more diversity in viral populations between individuals, allowing a greater insight into household transmission "chains". NGS is also being used to study transmission chains of H3N2 viruses in Hong Kong SAR. The centre is undertaking similar studies in other settings, such as schools, to determine which activities are associated with higher transmission risk. Because haplotyping was identified as a problem with NGS, the centre switched to PacBio to phase SNPs identified from short-read data, to determine which reads were in agreement between the platforms.

5.1.8 OFFLU

A preliminary survey on NGS use at OFFLU laboratories was conducted. Twelve laboratories participated, of which 10 were international reference laboratories of the OIE/FAO and two were regional laboratories. Of these laboratories, 57% used NGS for influenza A detection or

characterization (or both). Also, 67% have an NGS platform available, of which 80% were MiSeq, 10% were MiniSeq and 10% were Ion Torrent. In addition, 63% of laboratories used NGS as a primary diagnostic test and for viral characterization, whereas 37% used NGS solely for characterization. Most of the samples analysed by NGS were swabs (36%), isolates (32%) and organs or tissues (20%). The NGS turnaround time for influenza A virus detection or characterization was at least 3 days. Protocols were adapted from the Zhou protocol. It was noted that further discussion and evaluation were needed to identify more adequate NGS workflows for the OFFLU network.

5.1.9 Public Health Laboratory Centre, Department of Health, Hong Kong SAR, China

The workflow for viral characterization performed at the centre mainly involved Sanger sequencing (because of the low throughput requirement), and RT-PCR followed by electrophoresis, cycle sequencing, cycle sequencing clean-up then NGS, for which data analysis could usually be performed the following day. Concerns regarding NGS were presented; for example, in comparison to Sanger sequencing, NGS requires PCR clean-up steps, library preparation and specific bioinformatics resources. NGS is also relatively expensive compared with Sanger sequencing, and requires specific techniques and training. Further, NGS can take up to a week to complete, whereas Sanger sequencing takes about 2 days.

5.2 Guidance to GISRS on the use of NGS

NGS is considered an important issue among European NICs. With more laboratories purchasing NGS platforms, more are asking WHO for guidance. This was discussed at the fifth joint WHO/Europe and European Centre for Disease Prevention and Control (ECDC) Meeting on Influenza Surveillance in Budapest, Hungary, 14–16 June 2016. Topics discussed included the rationale for conducting NGS in influenza surveillance, the pros and cons for NICs of implementing NGS compared with Sanger sequencing, and what support would be needed from ECDC or WHO/Europe. Regarding the rationale for conducting NGS, most participants said that collecting complete sequence data and maintaining a repository of sequences would allow more insightful, in-depth analyses to be conducted, including better characterization of the virus population diversity for both currently circulating, and new and emerging viruses. Challenges in the timely and automated analysis of big sequence data were discussed and were identified as one limitation of NGS implementation for the whole network.

Implementation of NGS should begin in NICs, where resources are available to establish such capacity. The rapid development of the technology with different platforms and software solutions does not allow a recommendation of one particular system at the moment. However, it was recommended that some NICs pilot the introduction of NGS using different systems. Action points identified were to:

- identify objectives and describe outputs for the use of NGS in influenza surveillance;
- further facilitate work and discussion around implementation of NGS; and
- continue to provide support for Sanger sequencing and NGS through training and twinning with laboratories using the same technology (platforms) and algorithms.

Training for NGS was considered difficult and time consuming, and troubleshooting was also a potential problem, particularly if trainees did not return to and continue to work in their host laboratories. It was thought that NGS should not replace Sanger sequencing in NICs unless technology such as MinION was feasible. There was also discussion about the appropriateness of NGS at NICs, and concerns that if laboratories began using NGS without adequate preparation then they would not produce good data. To this end, one of the action points identified during the VCM in September 2016 was the development of guidance for NICs wanting to perform NGS. It was thought that the PCR WG could be tasked with providing guidance and information about RT-PCR and other technologies to laboratories.

5.3 Way forward

5.3.1 Strategy for GISRS virus detection
For seasonal surveillance, rRT-PCR is still considered the method of choice for virus detection and diagnosis. All NICs should be able to subtype seasonal influenza A viruses, and are encouraged to develop capacity for lineage determination of influenza B viruses. Further, singleplex rRT-PCR is still the "gold standard" in most laboratories. Multiplexing was discussed, but one of the challenges is that it involves proprietary equipment, which is more restrictive for the end user owing to the types of fluorophore available. Therefore, care needs to be taken, because products from different companies may not be comparable even if they involve the same fluorophores.

Any influenza A viruses that cannot be subtyped can be classified as novel (unsubtypeable) viruses. However, usually these are seasonal influenza A viruses with high Ct values rather than actual novel viruses. Most NICs understand the difference between unsubtypeable and non-subtyped viruses, but there were concerns regarding the NIC terms of reference covering unsubtypeable viruses. The guidance for NICs states that unsubtypeable viruses must be sent to CCs as soon as possible. However, the IHR articles were not clear regarding the sharing of viruses. It was recommended that, even if the viruses were subtyped by the NIC, they should still be sent to a CC.

5.3.2 GISRS capacity: gaps and priority actions
- It was recommended that the next EQA should include a survey to obtain more information from NICs (e.g. on the origins of the protocol being used) and collect data on GISRS capacity.
- Strengthening of virus detection capacity was highlighted, with the goal of ensuring that all countries have the capacity to detect events, collect specimens and ship them to the CCs. This will help to identify and prioritize aspects of the network that need to be improved.
- A deadline of the end of May was recommended for CCs and participating institutions to inform the WG about whether any RT-PCR protocols need to be revised.
- The possibility of a revision of the name of the PCR WG was raised. It was noted that a high-level terms of reference statement will be developed (to ensure that the WG is visible), and participants were encouraged to think about a possible new name.

5.3.3 EQAP
Laboratories are encouraged and expected to provide subtype information on samples. To this end, it was suggested that, from the next panel onwards, all seasonal viruses be subtyped correctly to obtain a score on the EQAP and to inform laboratories of this impending change. In the case of samples for antiviral testing, the subtypes of the samples will be revealed to the laboratories.

5.3.4 Use of NGS
It was agreed that guidance regarding the use of NGS, including Dr Deng's presentation, will be made available. It was agreed that although a "sequence first" strategy is being developed for influenza surveillance, in reality, protocols will remain rRT-PCR first followed by sequencing.

5.3.5 Development and update of guidance for NICs
The detection of A/H5 is a basic requirement for NICs and H5 reference laboratories. Revision is needed because the requirement for H5 reference laboratories has declined significantly since their establishment in 2003–2004. However, because the terms of reference for H5 laboratories are in the pandemic influenza preparedness (PIP) framework, they cannot be removed. Further discussion is needed to determine whether the list of H5 reference laboratories on the WHO website should continue to be maintained. It was also suggested that the guidance for laboratories are updated to include subtype A/H7 detection capacity.

5.3.6 Roles, responsibilities and functions of the GISRS and the PCR WG
In light of the huge technological advances since the PCR WG was founded, the future role and

goals of the group were discussed. It was suggested that the WG could engage more with experts, and that the role of the chair be extended beyond the meetings, to ensure that actions identified are acted upon. Any updates to protocols should be disseminated via EZCollab, and it was suggested that protocol changes be highlighted, together with provision of contact information and a paragraph containing limited information, to make it easier for laboratories to implement up-to-date procedures.

5.3.7 Possible publication in peer-reviewed journals

The possibility of publication of the efforts of the PCR WG in a peer-reviewed journal was discussed, which led to further discussion of the role of the WG. The purpose of the WG was originally to provide capabilities to NICs and other laboratories to detect emerging viruses such as A(H5N1). It was then extended into providing technical support for rRT-PCR in general. Originally, the WG provided protocols and control materials to help laboratories understand how different A/H5 assays performed, and whether they could detect circulating A/H5 viruses. The role of the WG then evolved to the provision of reagents and training, and to improving quality and capacity. The WG served to coordinate the actions of the numerous independently acting support systems, notably during the 2009 pandemic, to form a coordinated response that has now been extended to other viruses with zoonotic or pandemic potential (e.g. A(H5Nx), A(H7N9), A(H9N2)).

6.0 Proposed action points

The outcomes of the meeting have been published in an executive summary in the Weekly Epidemiological Record (vol. 92, 41; pp. 609–624)[7].

- rRT-PCR protocols on the WHO website should be reviewed to ensure they are current (deadline for updates is the end of May).
- Multiplex PCR protocols should be provided if relevant (Dr Hungnes is to be contacted for more information about protocols).
- NGS advice to NICs (Dr Daniels to contact Dr Wentworth and Dr Garten about this).
- Preparation of a manuscript based on the activities of the PCR WG over the past 10 years (Dr Lindstrom and Dr Zanin to draft the manuscript).
- The 2018 EQAP panel should be modified to score for subtyping of seasonal influenza A viruses and detection of any non-seasonal influenza A viruses, and an A(H7N9) virus. Future GLP surveys should be standardized, if possible, for continuity of interpretation.
- The feasibility of involving OFFLU regional laboratories in the EQAP to be investigated.
- Updated PCR protocols on the WHO website to include a table of contents with hyperlinks.
- WHO to rename the WG to include other molecular technologies (e.g. the WHO WG on Molecular Technologies for Detecting, Subtyping and Characterization of Influenza).

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[7] http://apps.who.int/iris/bitstream/10665/259233/1/WER9241.pdf?ua=1

Annex 1: List of Participants

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Annex 2: Declarations of interest

The 9th WHO WG Meeting on PCR Protocols for the Detection of Influenza A Virus subtypes, 12–13 April 2017, was organized by the GISRS and the Influenza Viruses and Vaccine Support team of WHO, with participation from WHO CCs on influenza, H5 reference laboratories, national influenza centres (NICs) and a representative from the veterinary sector – from the World Organization for Animal Health/ Food and Agriculture Organization of the United Nations (OIE/FAO) Network of Expertise on Animal Influenza (OFFLU).

In accordance with WHO policy, all the PCR WG expert members completed the WHO form for Declaration of Interests for WHO Experts before being invited to the meeting. These declarations were then evaluated by the WHO Secretariat before the meeting. At the start of the meeting, the interests declared by the expert members were disclosed to all consultation participants. The participants declared the following personal current or recent (within the past 4 years) financial or other interests relevant to the work of the group:

Institution	Representative	Personal interest
WHO CC London, United Kingdom	Dr Rod Daniels	None
WHO CC Melbourne, Australia	Dr Yi-Mo Deng	None
WHO CC, St Jude, US	Dr John Franks	None
WHO CC, St Jude, US	Dr Mark Zanin	None
WHO CC Japan	Dr Tsutomu Kageyama	None
WHO CC Atlanta, US	Dr Stephen Lindstrom	Patent inventor for PCR primers and probes ($4000)
The University of Hong Kong, Hong Kong SAR, China	Dr Leo Poon	None
The University of Hong Kong, Hong Kong SAR, China	Dr Daniel Chu	None
Department of Health, Hong Kong SAR, China	Mr Peter Cheng	None
Department of Health, Hong Kong SAR, China	Dr Janice Lo	None
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Department of Health, Hong Kong SAR, China	Mr Wong Chi Sun	None
Department of Health, Hong Kong SAR, China	Dr Irene Hui	None
EU/IRP	Dr Dmitriy Pereyaslov	None
Chinese CDC	Dr Xiang Zhao	None
Instituto Zooprofilattico Sperimentale delle Venezie, Padua, Italy	Dr Isabella Monne	None

Based on WHO assessment of the interest declared by Dr Lindstrom, it was concluded that Dr Lindstrom had no conflict of interest with the objectives of the technical consultation. Dr Lindstrom therefore participated in the meeting as an expert working group member.

9th WHO Working Group Meeting on RT-PCR for the Detection and Subtyping of Influenza Viruses

Public Health Laboratory Centre, Centre for Health Protection (CHP), Department of Health (DH), Hong Kong SAR, China
12 - 13 April 2017

Final Agenda

Wednesday, 12 April 2017   **Chair: J. Lo**

09:00 - 09:30	Welcome and opening	**J. Lo (CHP, DH, Hong Kong SAR, China)**
		W. Zhang (WHO
	Declaration of Interests Selection of chair Appointment of rapporteur	
09:30 - 09:40	Objectives and expected outcomes	**T. Besselaar**
9:40 – 10:30	**Session A: Review of actions since last meeting**	*Session co-chair T. Kageyama*
09:40 – 09:50	Recap of recommendations by the 2015 WG meeting	**T. Besselaar**
09:50 – 10:30	General updates from participating laboratories (10' each)	**Representatives from participating labs**
10:30 - 11:00	*Coffee break and photo*	
11:00 - 11:40	General updates from participating laboratories (cont.)	
11:40 - 11:55	Presentation PCR related activities in OFFLU (15')	**I. Monne**
11:55 – 12:30	Discussion	**All participants**
12:30 -13:30	*Lunch*	
13:30 – 15:30	**Session B: Quality assurance**	*Session co-chair S. Lindstrom*
13:30 – 14:00	EQAP: Observations on the progress made and future plans	**J. Lo**

14:00 – 14:20	Experience and lessons on learnt from the CDC quality assurance programme for PCR and future plans	**S. Lindstrom**
14:20 – 14:40	Experience and lessons on learnt from the EURO quality assurance programme for PCR and future plans	**D. Pereyaslov**
14:40 – 15:00	OFFLU strategy on external quality assurance for PCR	**I. Monne** **All participants**
15:00 – 15:30	Discussion	
15:30 - 16:00	***Coffee break***	
16:00 – 17:30	**Session C: PCR protocols for GISRS**	*Session co-chairJ. Franks*
16:00 – 16:30	Overview of current PCR protocols for GISRS	**T. Kageyama**
16:30 – 17:30	Discussion on gaps and actions	**All participants**
17.30	Close of day 1	**Chair**

Thursday, 13 April 2017

09:00 – 09:15	Summary of Day 1	**Chair**
09:15 – 12:30	**Session D: Next Generation Sequencing and other emerging molecular technologies**	**Session co-chair L. Poon**
09:15– 09:25	Overview of the emerging technology development	**Y. Deng**
09:25 – 10:25	Use of NGS in individual institutions: (10 min each): • CDC, Atlanta • CNIC, Beijing • Crick, London • VIDRL, Melbourne • St. Judes, Memphis • NIID, Tokyo	
10:25 - 10:50	***Coffee break***	
10:50 – 11:20	Use of NGS in individual institutions (cont.) • HKU, Hong Kong SAR, China • OFFLU • CHP, Hong Kong SAR, China	
11:20 –	Discussion: Guidance to GISRS on using NGS	

12:30

12:30 - 13:30	***Lunch***	
13:30 – 17:00	**Session E: Way Forward**	*Session co–chair* *R. Daniels*
	Discussions: 1) Strategy for GISRS virus detection • Novel viruses • Seasonal surveillance 2) GISRS capacity: gap and priority actions 3) EQAP 4) Use of NGS 5) Development and update of guidance for NICs 6) Roles, responsibilities and functions of GISRS PCR WG 7) Possible publications in peer reviewed journal	**All participants**
15:00 - 15:30	***Coffee break***	
17:00 - 17:20	Summary	**Chair**
17:20 – 17:30	Summary and closure	**W. Zhang**

References

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